



# Proteomic and Ultrastructural Analyses of Human Lipofuscin

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## Abstract

**Purpose:** The progressive accumulation of lipofuscin in the retinal pigment epithelium (RPE), correlates with the pathogenesis of age-related macular degeneration (AMD). We seek a better molecular understanding of the sources and consequences of lipofuscin accumulation, including the protein content of lipofuscin.  
**Methods:** Human RPE lipofuscin was purified by conventional sucrose density gradient centrifugation methods. Lipofuscin granule purity was evaluated by light, fluorescence, confocal, and electron microscopy. Lipofuscin preparations were extracted with chroform/methanol then the chroform insoluble material was extracted with SDS and subjected to SDS-PAGE, gel bands excised and proteins identified by LC MS/MS. Western analysis was used to probe for oxidative protein modifications.  
**Results:** Ultrastructural analyses of lipofuscin purified by conventional methods revealed a heterogeneous core structure composed of lipofuscin granules surrounded by substantial extra-granular material. The chroform insoluble lipofuscin fraction of the conventional preparation exhibited many fuzzy Coomassie blue stained SDS-PAGE bands, suggesting post-translational modifications. Western blot analysis confirmed the presence of abundant carboxytryptophan adducts. Over 160 proteins were identified, ~33% of which exhibited apparent mass additions. Essentially "pure" lipofuscin granules, free of extra-granular material, were obtained by proteolytic digestion of the conventional preparation. Boiling the purified granules in SDS has so far failed to yield SDS-PAGE detectable bands with Coomassie or silver staining.  
**Conclusions:** Lipofuscin granules appear to be embedded in a protein "matrix" in content in drusen. Proteomic characterization of purified lipofuscin granules is underway.

CR, N. Supported in part by NIH grants EY16063, EY14245, EY15882, HL33145, HL35315, Fight for Sight, The Foundation Fighting Blindness, The Cleveland Clinic Foundation and The Wellcome Trust, UK.

## Introduction

Lipofuscin is a heterogeneous group of lipid/protein aggregates that have a characteristic yellowish-blue-green fluorescence when excited with ultraviolet light (1). These lipofuscin inclusions are located within lysosomal debris and accumulate with age in a variety of postmitotic cells as non-degradable end products (1, 2). Formation of lipofuscin in the retinal pigment epithelium (RPE) appears to be closely associated with retinoids and a normally functioning visual cycle. RPE lipofuscin accumulation also correlates with age-related macular degeneration (3,4) and as its accumulation may represent an active stage in the progression of the disease. We and others have proposed that oxidative modification of cellular proteins and unrelated proteins that simply co-migrate with fluorescence in sucrose density gradient isolation procedures. Here we report proteomic and ultrastructural analyses of three lipofuscin preparations, which vary in lipofuscin granule purity and in donor age.

## Methods

**RPE Lipofuscin Sample Preparation:** Human eyes were obtained from UK tissue resources and RPE cells isolated and kept frozen at -70°C until sufficient material was collected for isolation of lipofuscin granules. RPE lipofuscin preparations were isolated from 60-70 y donors, 50-60 y donors, and 70-90 y donors. Lipofuscin granules were isolated by mechanical homogenization and separation by using a narrow gauge needle (5) followed by differential low speed centrifugation to obtain a crude suspension of granules followed by up to three high speed discontinuous sucrose density gradient centrifugations (2.0–0.3M). Isolated granules from the 70-90 y donors were further treated with protease K (10 µg/µl, 24 hours temperature) in 15 mM N-methyl morpholine acetate pH 8.2, 1 mM EDTA, 100 µM BHT, 0.2% SDS, then washed with buffer without SDS prior to analyses. Granules were quantified by counting on a hemocytometer and purity was evaluated by light, fluorescence, confocal, and electron microscopy. All three lipofuscin preparations were extracted with chroform/methanol (2:2 v/v) and the chroform insoluble material boiled in SDS sample buffer and subjected to 1D SDS-PAGE.

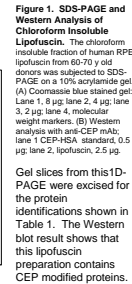
**Light and Confocal Microscopy:** Light microscopy was performed with a Zeiss Axiophan Microscope. For confocal microscopy (6), lipofuscin preparations were mounted with Vectashield under a coverslip and analyzed using a Leica laser scanning confocal microscope (TCS-SP2, Leica, Exton, PA). Autofluorescence was carried out in the FITC (green), TRITC (red) and Cy5 (far red) channels. A series of 1 µm optical sections was collected. Each individual optical image of the granules represents a three-dimensional projection of the entire cryosection (sum of all images in the stack). Microscopic panels were composed using Adobe Photoshop CS5.

**Transmission Electron Microscopy:** Lipofuscin preparations were pelleted in an eppendorf tube, and fixed in a mixture of 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1M cacodylate buffer. The pellet was washed in the same buffer, post-fixed in 1% OsO<sub>4</sub>, sequentially dehydrated in ethanol then embedded in Epon. Transmission electron micrographs were taken on a Tecnai 20, 200 kV digital electron microscope using a Gatan Image filter and digital camera at 3000 diameters (8).

**SDS-PAGE and Western Analysis:** Lipofuscin preparations were subjected to 1D SDS-PAGE according to Laemmli on 10% gels and stained with Coomassie blue (Pierce Code Blue) for proteomic analysis. Gel slices (~2 mm) were excised from the top to the bottom of the lane, digested in-situ with trypsin, and peptides extracted for LC MS/MS. Western analysis for carboxytryptophan (CYP) modifications was performed with monoclonal anti-CYP antibody (7).

**Protein Identification:** LC MS/MS was performed with a CapLC system and a quadrupole time-of-flight mass spectrometer (QTOF) (8). Protein identifications from MS/MS data utilized ProteoMiner/MSI Global Server (Waters Corporation) and Mascot (Matrix Science) search engines and the Swiss-Prot and NCBI protein sequence databases.

**Amino Acid Analysis:** Lipofuscin granules from human eyes were subjected to vapor phase HCl hydrolysis and phenylthiocarbonyl (PTC) amino acid analysis (8) using Agilent 1100 HPLC system with autoinjector, a Gilson 116 UV detector and an ABI 112A oven.



**Figure 1. SDS-PAGE and Western Analysis of Chloroform Insoluble Lipofuscin.** The chloroform insoluble fraction of human RPE lipofuscin from 60-70 y old donors was subjected to SDS-PAGE on a 10% acrylamide gel. (A) Coomassie blue stained gel. Lane 1, 8 µg; lane 2, 4 µg; lane 3, 2 µg; lane 4, molecular weight markers. (B) Western analysis with anti-CYP antibody. Lane 1 CYP-HSA standard, 0.5 µg; lane 2, lipofuscin, 2.5 µg. Gel slices from this 1D-PAGE were excised for protein identifications shown in Table 1. The Western blot result shows that this lipofuscin preparation contains CYP modified proteins.

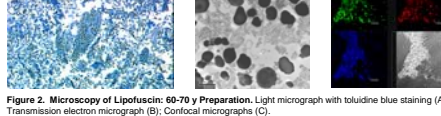
Protein ID <sup>a</sup>	Accession No. <sup>b</sup>	Peptide Matches	Calc. mass (kD)	Observed <sup>d</sup> mass (kD)
Actin, Beta	P60709	7	42	132,118,105,85,65,42,37,33,20,28,23,21,18,16,15,13,12,9
ADP/ATP carrier protein	P05141	5	35	148,132,118,105,85,65,42,37,33,20,28,23,21,18,16,15,13,12,9
Glyoxalase I (glyoxalase 1)	P04468	12	38	148,118,105,85,65,42,37,33,20,28,23,21,18,16,15,13,12,9
Glyoxalase II (glyoxalase 2)	P11468	7	47	132,118,105,85,65,42,37,33,20,28,23,21,18,16,15,13,12,9
Retinol epithelium protein 15	Q18618	5	61	148,95,83,23,23,18,13,12,9
Ribonuclease (Pan 2)	P08100	4	39	148,132,118,105,85,65,42,37,33,20,28,23,21
Retinol epithelium protein 11 c1b	Q20791	7	36	132,118,105,85,65,42,37,33,20,28,23,21,18,16,15,13,12
Cdkal1, retinoblastoma-binding protein (CRABP1)	P12271	5	38	118,105,83,23,23,21,18,16,15,13,12
Oxalate hydratase, H-Chaper	P12277	12	43	118,42,29,28,18,16,15,12
Isocitrate dehydrogenase (NADP+)-dependent	P12827	12	112	148,83,69,29,20
Cathepsin D	P07339	8	46	148,132,28,23,21,18
Protein tyrosine phosphatase 1B (SH-PTPase)	P08719	4	102	148,132,118,105,85,65,42
RPE retinal G-protein-coupled receptor	N07014	2	32	66,59,23,21,15,13
Glyoxalase III (beta)	P08124	4	23	152,16,15,13,12
Protein tyrosine phosphatase 1B (SH-PTPase)	Q20792	37	118,105,26,26,21	
Actin, alpha isoform	P02568	7	42	37,33,13,12
Cystatin B, Beta	P03320	4	23	148,13,13,13
Green-sensitive opsin 1 (Green photostop pigment)	P52589 (G)	2	41	148,118,105,34
Membrane-associated retinol-binding protein (MRBP)	P17146	1	23	118,102,35,16
Mitochondrial 5-oxoglutarate/oxaloacetate transaminase (OGOT)	Q20793	6	34	23,21,18,16
S-arabinoside (Rafinofin S)	P10325	4	46	74,42,23,21
Isocitrate dehydrogenase (NADP+)-dependent	P05028	8	102	148,132,118,105,85,65,42
Cathepsin D	P29284	4	88	132,74,66
Cystatin B, chain, Alpha	P02511	3	20	13,12,8
Enolase, Alpha	P06733	2	47	29,18,10
Histone H4	P02034	9	11	10,5,8
Phosphatase carrier protein (PTP)	P05028	2	40	74,23,21
Annexin V	P06758	2	38	23,5
Cystatin A, Beta	P10373	1	20	16,13
Cystatin S, Gamma	P20914	1	21	15,13
Histone H2B.1	P10778	3	14	9,8
Histone H2B.2	P06756	5	18	10,8
Cystatin S, Gamma	P20914	1	21	15,13
Histone H2B.1	P10778	3	14	9,8
Histone H2B.2	P06756	5	18	10,8
Lysosome membrane protein 1	Q14108	4	54	83,74
Neutrophil cathepsin release 1, 10 kD isoform	P125905	2	117	132,105
Oxoprolinase (retinol dehydrogenase-like protein)	A05860	3	32	21,18
Protein tyrosine phosphatase (non-receptor class protein)	P29282	2	39	29,23
Neutrophil cathepsin release 1, 10 kD isoform	P125905	2	117	132,105
Annexin A2	P06749	2	20	13
Cystatin A, Beta	P02511	1	20	13,12,8
Cystatin S, Beta	P05074	2	26	10
Phosphatidylethanolamine-binding protein	P03086	1	21	15
Protein	P02529	3	30	18
Ribonuclease protein 3	Q06197	2	35	15
Cathepsin D (non-receptor class protein)	Q21273	1	35	15

<sup>a</sup>Protein was identified by LC MS/MS from SDS-PAGE fractionated chloroform insoluble lipofuscin from 60-70y donors. About 28% of the proteins identified had 4 or 5 tryptic peptides. Many of these proteins exhibited substantial apparent mass additions suggestive of posttranslational modifications including protein oxidation.

<sup>b</sup>Swiss-Prot database accession numbers are shown in plain font.

<sup>c</sup>Based on protein sequence.

<sup>d</sup>SDS-PAGE observed mass.

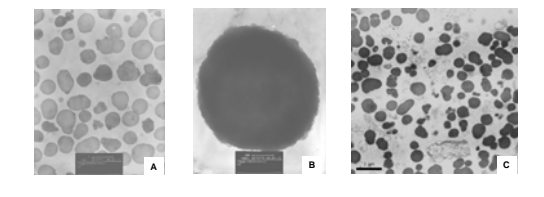


**Figure 2. Microscopy of Lipofuscin: 60-70 y Preparation.** Light micrograph with toluidine blue staining (A); Transmission electron micrograph (B); Confocal micrographs (C). This figure shows significant extragranular material in the 60-70 y lipofuscin preparation. Select proteins identified from this preparation are shown in Table 1. For proteomic characterization of lipofuscin, this preparation raised the question "What is authentic lipofuscin – only the granules, or does lipofuscin include the material between the granules?" We subsequently sought "pure" lipofuscin granules for proteomic analysis.

AA	50-60y Lipofuscin <sup>a</sup>		70-90y Lipofuscin <sup>a</sup>	
	Average Amount (pmol ± SD)	Mol % ± SD	Average Amount (pmol ± SD)	Mol % ± SD
Asp	141.3 ± 2.5	6.40 ± 0.12	20.6 ± 0.5	4.30 ± 0.16
Glu	113.3 ± 1.7	5.13 ± 0.06	30.9 ± 1.1	6.45 ± 0.06
Ser	128.4 ± 5.7	5.85 ± 0.25	47.4 ± 2.2	11.80 ± 0.19
Gly	220.2 ± 0.8	9.98 ± 0.04	37.5 ± 1.0	7.82 ± 0.06
His	35.1 ± 1.2	1.59 ± 0.06	6.5 ± 0.1	1.37 ± 0.04
Arg	58.9 ± 0.3	2.67 ± 0.03	13.2 ± 0.9	2.76 ± 0.12
Val	124.3 ± 8.6	5.63 ± 0.17	17.5 ± 1.7	3.61 ± 0.25
Ala	158.2 ± 4.4	7.17 ± 0.22	28.7 ± 0.9	5.98 ± 0.11
Pro	128.4 ± 1.8	5.82 ± 0.07	11.7 ± 1.4	2.38 ± 0.40
Tyr	227.1 ± 5.2	10.29 ± 0.17	35.9 ± 0.8	7.50 ± 0.26
Met	99.1 ± 4.4	4.49 ± 0.19	11.2 ± 1.2	2.32 ± 0.20
Ile	163.6 ± 0.6	7.41 ± 0.07	39.4 ± 1.3	8.22 ± 0.11
Leu	332.2 ± 1.2	15.05 ± 0.12	50.5 ± 1.2	10.53 ± 0.31
Thr	109.2 ± 1.1	4.95 ± 0.08	17.5 ± 1.0	3.62 ± 0.12
Lys	62.3 ± 3.5	2.82 ± 0.14	42.2 ± 1.3	8.79 ± 0.11
Total	2207.2 ± 14.4	100	479.5 ± 14.0	100
Granules hydrolyzed <sup>b</sup>	1.0 × 10 <sup>6</sup>		1.04 × 10 <sup>6</sup>	
Granules analyzed <sup>c</sup>	20.2 ± 10 <sup>6</sup>		7.3 ± 10 <sup>6</sup>	
Granules/µg AA <sup>d</sup>	81 × 10 <sup>6</sup>		4.1 × 10 <sup>6</sup>	

<sup>a</sup>Lipofuscin from 50-60y donors was subjected to vapor phase HCl hydrolysis and phenylthiocarbonyl (PTC) amino acid analysis (n=9).  
<sup>b</sup>Lipofuscin from 70-90 year old human eyes was treated with protease K prior to vapor phase HCl hydrolysis and PTC amino acid analysis (n=4).  
<sup>c</sup>Granules were counted using a hemocytometer.

## Results



**Figure 3. Microscopy of Lipofuscin: 50-60 y Preparation.** Transmission electron micrographs of two different sections from the 50-60 y preparation of lipofuscin granules (A, C) and for a single granule (B).

Much less extragranular material is apparent in the 50-60 lipofuscin preparation as compared to that in Figure 2. Select protein identified from this preparation are shown in Table 2.

Table 3. Proteins Identified in Human RPE Lipofuscin, Chloroform Insoluble Fraction, 50-60y donors.

Protein ID <sup>a</sup>	Accession No. <sup>b</sup>	Peptides Matches	Calc. mass (kD)	Observed <sup>d</sup> mass (kD)
Myelin proteolipid protein 1	P06201	6	30	10,22,28,30,40,50,60,72,88,114,130,140,150
Histone H2B.2	Q20980	2	14	15,22,28,30,35,48,60,72,95,114,140
Histone H2B.1	P13778	2	14	15,22,28,30,35,48,60,72,95,114,140
Actin ceramide precursor	Q13510	7	45	15,22,28,30,33,34,35,37
Cathepsin D precursor	P07339	10	45	10,22,28,30,33,34,35,150
GTP-binding nuclear protein Ran	P62205	25	25	28,30,40,48,60,80,100,120,140
Histone H4	P62805	30	11	15,22,28,32,32,140
Ig kappa-1 chain C region	P01857	1	36	17,19,22,24,33,60
Myelin basic protein	P02886	1	33	15,17,19,26,33,60
Rhodopsin	P08100	2	39	15,17,19,22,37
Sodium/potassium-transporting ATPase beta-1 chain	P05226	1	35	15,26,30,32,33,34
Succinate dehydrogenase	P21912	1	32	19,48,50,60,95
Serum albumin precursor	P02768	5	69	46,48,50,88
Tripeptidyl-peptidase I precursor	Q14773	1	61	15,17,19,22
Green-sensitive opsin	P04001	1	41	17,50,140
Nuclear pore complex protein Nup88	Q09567	1	84	15,17,19
Annexin A2	P07355	2	38	25,28
Calnexin precursor	P27824	1	67	24,25
Hemoglobin beta chain	P06871	2	16	10,15
Ig kappa chain C region	P01834	1	12	15,22
Integrin alpha-V precursor	P06756	2	17	22,24
Lysosome C precursor	P13625	1	24	140,150
Metalloproteinase inhibitor 3 precursor	Q89104	8	45	35,42
Squamous cell carcinoma antigen 1	Q9UKV4	2	98	22,28
Vav-3 protein	Q96A52	7	44	32
Histone H43	P68431	7	15	17
HSD-33	Q6W998	11	46	15
immunoglobulin heavy chain variable region	Q2P1J4	2	52	40
Junction plakoglobin	P14923	3	81	29
Lysosome membrane protein II	Q14108	1	54	33
NipSnap1 protein	Q8BPW8	2	33	19
Novel protein similar to histone 2	Q5TEC6	15	15	15
Phakiosin	Q15151	3	62	29

<sup>a</sup>Proteins were identified from by LC MS/MS from SDS-PAGE fractionated chloroform insoluble lipofuscin from 50-60y donors. About 44% of the proteins identified are shown (34 of 78 total). Apparent mass additions are suggestive of posttranslational modifications, including oxidative protein modifications and cross-links.  
<sup>b</sup>Swiss-Prot database accession numbers are shown in plain font.  
<sup>c</sup>Based on protein sequence.  
<sup>d</sup>SDS-PAGE observed mass.

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Table 4. Proteins Identified in Human RPE Lipofuscin, Chloroform Insoluble Fraction, 70-90y donors.

Protein ID <sup>a</sup>	Accession No. <sup>b</sup>	Peptide Matches	Calc. mass (kD)	Observed <sup>d</sup> mass (kD)
Beta crystallin B2	P43320	20	23	21,22,24,30,31, 34,38,40, 42,47,49,51,57, 63, 71,77,83,85,120,130,140,150
Beta crystallin A3	P06183	8	25	21,22,30,31,33,34,38,39,40,41,42,47,50,59,101,119,150
Beta crystallin A4	P56873	13	22	21,30,31,33,34,38,39,40,41,42,47,50,59,101,119,150
Lysosome C	P01526	1	16	32,33,34,35,36,38,40,44,46,48,50,55,60,50
Ig kappa chain C region	P01834	1	11	32,33,34,35,40,50,60,65,88,140
Origin recognition complex subunit 4	Q43929	2	50	22,30,32,33,120,140,145
Beta crystallin B1	P50674	7	28	22,30,31,33,34,35,42
Alpha crystallin A chain.	P02489	9	20	30,33,34,43,114
Beta crystallin B3	P26968	12	24	30,32,33,34
Ig kappa chain C region	P01877	2	36	36,38,65
Ig alpha-1 chain C region	P01876	2	37	35,36,44
Beta crystallin S3	P22914	9	21	21,30,33
Retinoic acid receptor RXR-alpha	P19793	1	92	80,85
Serum albumin precursor	P0			